Antidepressant-like Activity of Solvent Fractions of the Root Bark of *Carissa spinarum* Linn. (Apocynaceae) in Rodents

By

Hana Saif Ali

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This is to certify that the thesis prepared by Hana Saif Ali, entitled: "Antidepressant-like Activity of Solvent Fractions of the Root Bark of *Carissa spinarum* Linn. (Apocynaceae) in Rodents", and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

Internal examiner	**Dr. Workineh Shibeshi**	Signature	Date
External examiner	**Dr. Worku Bedada Waktola**	Signature	Date
Advisor	**Prof. Ephrem Engidawork**	Signature	Date

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Chair, Department or Graduate Program Committee

ABSTRACT

Antidepressant-like Activity of Solvent Fractions of the Root Bark of *Carissa spinarum* Linn. (Apocynaceae) in Rodents

Hana Saif Ali

Addis Ababa University, 2022

Depression is a chronic and recurrent psychiatric disorder affecting mental and physical health. Traditional medicines have been used for decades in the treatment of depression due to resistance development, side effects, inadequate accessibility and affordability of modern antidepressant drugs. The root bark of *Carissa spinarum* Linn. (Apocynaceae), which is used traditionally for depression is reported to possess antidepressant-like activity in rodent models of depression. In the current study, different fractions of the root bark obtained using solvents of different polarities were investigated using a host of models, including tail suspension test (TST), forced swim test (FST), muricidal behavioral test, and open field test (OFT). Moreover, markers for depression and phytoconstituents were determined, and preliminary mechanistic studies using different drugs were also conducted.

Five groups of animals of either sex each having 6 animals per group were randomly assigned for every model and each solvent fraction (Ethyl acetate, n-butanol, and aqueous). Group I, served as a negative control group and received a vehicle (2% Tween 80), group II served as a positive control and received the standard drug Imipramine (30 mg/kg), group III-V received increasing doses of the solvent fractions 50, 100 and 200 mg/kg, respectively. Ethyl acetate (CSE) and n-butanol (CSB) fractions significantly ($p<0.001$) reduced the duration of immobility in TST and FST, while the aqueous fraction (CSA) failed to show any appreciable antidepressant-like activity. No change in the locomotor activity using OFT was observed at all doses. From the two active fractions, the ethyl acetate fraction was the most effective and contributed to reducing the muricidal behavior in rats. Serum corticosterone level was reduced by both fractions and once again the ethyl acetate fraction was the most effective. Mechanistic studies revealed the involvement of

multiple neurotransmission systems, including adrenergic, dopaminergic and cholinergic as well as the L-Arginine-NO-cGMP pathway in the antidepressant-like effect of the plant, hence only both the dopaminergic and the L-Arginine-NO-cGMP pathway were the predominant by which the ethyl acetate fraction might mediate its action. Phytochemical analysis showed the presence of high content of alkaloids (0.17 mgATE/g and 0.07 mgATE/g), flavonoids (12.43 mgQE/g and 2.07 mgQE/g), and phenols (42.42 mgGAE/g and 29.8 mgGAE/g) for CSE and CSB, respectively. The findings collectively indicate that CSE and CSB are endowed with antidepressant-like activity due to the presence of phenols, flavonoids, and alkaloids, which are medium polar in nature.

Keywords: Depression, *Carissa spinarum* Linn., Antidepressant-like activity, Solvent Fractions, Forced Swim Test, Tail Suspension Test, Muricidal Behavioral Test, Open Field Test, Serum Corticosterone Level, Pharmacological drugs.

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Hana Saif Ali

Table of Contents

LIST OF ABBREVIATIONS

5-HIAA	5-hydroxy-indoleacetic acid
5-HT	5-hydroxy-tryptamine (serotonin)
AAU	Addis Ababa University
AD	Alzheimer's disease
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of Variance
BCG	Bromocresol Green
BDI	Beck Depression Inventory
BDNF	Brain-Derived Neurotrophic Factor
BP	British Pharmacopeia
CHD	Coronary Heart Disease
CNS	Central Nervous System
CORT	Cortisol
CRP	C-reactive protein
CSA	Aqueous fraction of *Carissa spinarum* Linn.
CSB	n-Butanol fraction of *Carissa spinarum* Linn
CSE	Ethyl acetate fraction of *Carissa spinarum* Linn
CSF	Cerebrospinal fluid
DA	Dopamine
DALYs	Disability-Adjusted Life Year
DBS	Deep Brain Stimulation
DM	Diabetes Mellitus
DMSO	Dimethyl Sulfoxide
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
ECLIA	Electrochemiluminescence Immunoassay
ECT	Electroconvulsive Therapy
FDA	Food and Drug Administration
FST	Forced Swim Test
GABA	Gamma Aminobutyric Acid
GBD	Global Burden of Disease

GDS	Geriatric Depression Scale
HAM-D or HRSD	Hamilton Rating Scale for Depression
HPA	Hypothalamic-Pituitary-Adrenal axis
ICD-10	The tenth International Classification of Diseases
IDS or QIDS	Inventory of Depressive Symptomatology
IL	Interleukin
IP	Imipramine
i.p.	Intraperitoneal
MADRS	Montgomery and Asberg Depression Rating Scale
MAOI	Monoamine oxidase inhibitors
NaSSAs	Noradrenergic and Specific Serotonergic Antidepressants
NDRI	Norepinephrine and Dopamine Reuptake Inhibitor
NE	Norepinephrine
NMDA	N-Methyl-D-Aspartate
NO	Nitric Oxide
NRI	Noradrenalin Specific Reuptake Inhibitor
OFT	Open Field Test
PD	Parkinson's disease
PMDD	Premenstrual Dysphoric Disorder
P.O.	Per oral
PPD	Postpartum Depression
RIMA	Reversible Inhibitor of MAO-A
rTMS	Repetitive Transcranial Magnetic Stimulation
SARIs	Serotonin-2 Antagonist/Reuptake Inhibitors
SEM	Standard Error of Mean
SNRIs	Serotonin and Norepinephrine Reuptake Inhibitor
SPSS	Statistical Package for the Social Sciences
SSRIs	Serotonin Selective Reuptake Inhibitor
SST	Serum-separating tube
TAC	Total Alkaloids Content
TCAs	Tricyclic Antidepressants

tDCS	Transcranial Direct Current Stimulation
TFC	Total Flavonoids Content
TNF-α	Tumor Necrosis Factor-Alpha
TPC	Total Phenols Content
TRD	Treatment Resistant Depression
Trk-B	Tropomyosin Receptor Kinase B
TST	Tail Suspension Test
USP	United State Pharmacopeia
UV	Ultraviolet-visible
VNS	Vagus Nerve Stimulation
VTA	Ventral Tegmental Area
WHO	World Health Organization
YLDs	Years lived with Disability
YLL	Years of Life Lost
Zung SDS	Zung Self-Report Depression Scale

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. Background

World Health Organization (WHO) defines mental health as "a state of well-being" in which any individual has the ability to be competent to cope, communicate and be productive with stress in life and/or any environmental changes (WHO, 2003). In contrary, mental health problem refers to behavioral, neurological, substance use disorders, and psycho-social disabilities (Scheid and Wright, 2017), which are determined by multiple contributions of social, economic, psychological, environmental, and biological factors (Vigo et al., 2016; WHO, 2004). Mental health disorder is universal and occurs in low, medium, and high levels of income societies with an estimation of 1.1 billion people being affected worldwide (Frankish et al., 2018; WHO, 2004, 2001).

Depression is one of the most common and serious brain disorders and is a chronic and recurrent psychiatric disorder affecting and changing mental and physical health. Also, it has a significant impact on healthcare resources and costs (Arnaud et al., 2021). It is a heterogeneous mental disorder with a psychological, behavioral, and physiological symptomatic manifestation, affecting one out of five individuals worldwide. It is also a multifactorial disease with different causes (Filatova et al., 2021; Surana and Wagh, 2018).

1.2. Epidemiology of depression

Mental disorders, among non-communicable diseases, have many roles in causing premature mortality. In 2016, depression is estimated to account for 14.3% of total deaths across the globe. About eight million deaths happen yearly, signifying death is attributable to mental disorders (Vigo et al., 2016).

As stated by WHO, depression is globally considered the major source for the overall growth in morbidity and disability. In effect, depression was ranked the second disorder in GBD (Vigo et al., 2016; WHO, 2017).

The disability-adjusted life year (DALYs) represents the sum of the years of life lost (YLL) due to premature mortality in the population and the years lived with a disability (YLD). Depression is believed to be the leading cause of disability worldwide for both sexes in all ages, ranked fourth in 1990 and 2000, and second in 2020, accounting for 12.3% and 15% of the DALYs lost, respectively (WHO, 2001). Recently, GBD showed that depression is one of the most two disabling mental disorders and a leading cause of burden worldwide in 2019 and is high across the entire lifespan for both sexes. Also, it has impacted mental health, where the highest prevalence is estimated to occur in sub-Saharan Africa, North Africa, and the Middle east (Santomauro et al., 2021), and a single most burdensome disorder during the middle years of life in both developing and developed countries (Wang et al., 2003).

WHO, in 2017, has estimated that globally, over 300 million people were suffering from depression, which was equivalent to 4.4% of the world's population. This number includes children, adolescents below 15, men, women, poor and wealthy individuals. Despite the occurrence in both sexes, it is common in females (5.1%) than males (3.6%) (WHO, 2017). Its prevalence varied according to the patient's age. Nevertheless, the highest peak of depression is seen in the elderly patients at the age of 80 and more, as a result of physical dysfunction, loss of personal control, and low life cycle. While it reaches the lowest level in the middle-aged, at 45 years old, as a result of the adjusted life cycle, accordingly, it is concluded that depression fall with increasing maturity and rise with low physical ability (Mirowsky and Ross, 1992).

Depression has an economic impact worldwide. Moreover, the association of depression with an enormous economic burden results mainly from the impaired work performance and partly from the widespread underuse and poor-quality use of efficacious and tolerable treatments. Despite the availability of effective antidepressants that improves clinical and low work productivity, only 50–60% of patients receive adequate treatment (Wang et al., 2003). With respect to economic status the rate of depression increases twice among the poor than the wealthy people, thus poverty highly contributes to the prevalence of depression (Kleinman, 2004; WHO, 2001). The World Economic Forum report estimated

that direct and indirect association costs of mental health disorders including depression were ~US$2.5 trillion and would pass US$6 trillion by 2030 (Bloom et al., 2012).

Depression is highly comorbid with other psychiatric disorders (i.e. anxiety disorders, substance abuse, somatic disorders ,and others.), which worsen the quality of life of patients, their families, and the society. (Sartorius, 2001). It also occurs with coronary heart disease (CHD), Alzheimer's disease (AD), and Parkinson's disease (PD) (Krishnan et al., 2002), diabetes mellitus (DM) (Katon, 2008), and some medical disorders such as migraines, insomnia, and irritable bowel syndrome (Bangasser and Valentino, 2014).

1.3. Types of depression

According to the *Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V)* and the *tenth International Classification of Diseases (ICD-10),* depression is classified into different subtypes. This classification has the primary role for appropriately choosing efficacious treatment and avoiding potential resistance. Accordingly, the diagnostic criteria for depression require the presence of five or more symptoms lasting two weeks of depression episodes. These symptoms are characterized by depressed mood, loss of interest or pleasure (anhedonia), weight loss or gain, appetite disturbances (increase or decrease), sleep disturbance (insomnia or hypersomnia), psychomotor agitation or retardation, fatigue, feeling of guilt or worthlessness, loss of concentration and functioning impairment, and ideation of committing or attempting suicide (Edition, F., 2013; Krishnan and Nestler, 2008; WHO, 1992).

According to the polarity of symptoms, depression is classified into unipolar and bipolar (Rahman et al., 2017). Unipolar depression has different subtypes, which are major depressive disorder and dysthymic disorder. Similarly, bipolar disorders have different subtypes, which are bipolar I depression (mania), bipolar II depression (hypomania), and bipolar III depression. Additionally, depression is classified according to its severity into mild (with/without somatic symptoms), moderate (with/without somatic symptoms), severe (with/without psychotic symptoms) and psychotic depression (mood-congruent or mood-incongruent) (Benazzi, 2006; Gilbert, 2016). Another classification based on the *Diagnostic and Statistical Manual of Mental Disorders*, 4th ed, Text Revision *(DSM-IV-*

TR) classifies depression based on the type of time-related specifiers for depression episodes. It is classified into catatonic, melancholic, atypical, and postpartum depression based on a series of cross-sectional specifiers; and into chronic, full inter-episode recovery (present or absent), seasonal, and rapid cycling depression based on longitudinal course specifiers (Benazzi, 2006).

Different types of depression are also highly associated to the interaction between biological and environmental factors. This includes reproductive depression, such as premenstrual dysphoric disorder (PMDD), postpartum depression (PPD), and perimenopausal depression, which develop due to hormonal fluctuation in the brain affecting neurotransmission (Osborne et al., 2016; Payne et al., 2009; Stewart and Vigod, 2019).

1.4. Pathophysiology of depression

The etiology and pathophysiology of depressive disorder continued to be unclear due to the multifactorial causes, such as the interaction of environmental, biological, psychological, and social factors (Moret and Briley, 2011; Payne et al., 2009). Various studies have proposed several hypotheses, which might contribute to explaining the major causes, mechanisms, and symptoms of depression. However, none are comprehensible since all share common pathways and roles in different ways and mechanisms. The hypotheses are the monoamine hypothesis, the hypothesis of stress-induced depression, the cytokine hypothesis, the neuroinflammation and neuroplasticity hypothesis, the GABA-glutamate-mediated hypothesis, the circadian hypothesis, and the cholinergic-monoaminergic interaction theory (Filatova et al., 2021).

The monoamine hypothesis: This hypothesis proposes that alteration of the monoaminergic neurotransmitters in the CNS, serotonin (5-HT), norepinephrine (NE), and/or dopamine (DA) play a major role in the pathophysiology of depression. The role of serotonin, which modulates motor function, pain perception, and appetite in the CNS, in depression was supported by studies indicating the reduction in the concentration of the major metabolite of serotonin, 5-hydroxy-indoleacetic acid (5-HIAA), reduction in the concentration of serotonin (5-HT) in different brain regions, reduction of the plasma

concentration of tryptophan, increased density of postsynaptic serotonin receptor binding sites and decreased number of serotonin transporters (Cowen, 2008; Owens and Nemeroff, 1994). Similarly, alteration of NE which regulates cognition, motivation, and intellect was seen in patients with depression (Moret and Briley, 2011). Moreover, alteration of dopamine, which modulates cognition and motor behavior, is linked to the reward system and also seen in patients with depression (Kapur and John Mann, 1992). Indeed, the currently available antidepressants act in accordance with this hypothesis and it is established in different preclinical and clinical studies. Depression that results from depletion of monoamines by reserpine and antidepressant like effect seen with iproniazid that increases monoamines concentration can be cited as evidence for this hypothesis (Brigitta, 2002; Filatova et al., 2021; Moret and Briley, 2011). However, the lack of antidepressant effect of amphetamine and cocaine, which also increase monoamine concentration cast doubt on the validity of this hypothesis (Brady et al., 2005; Gnegy, 2012)

Stress-induced depression hypothesis: explained that chronic stress and malfunction of the hypothalamic-pituitary-adrenal (HPA) axis contribute to the pathophysiology of depression. Hyperactivity of the HPA axis with prolonged exposure to stress leads to prolonged alteration and results in the development of adrenal hypertrophy and thymic atrophy, which are mainly associated with high corticotropin, and an increment of the glucocorticoid hormone. This in turn mediates long-term changes in the brain resulting in long-term memories of the stressful experience causing stress-related psychopathologies such as depression. (Doczy et al., 2009).

The Neurotrophic hypothesis: Neurotrophic factors regulate the neural growth and differentiation during development and these are potent regulators of plasticity and survival of adult neurons and glia. Neuronal plasticity is the ability to acquire information and make appropriate responses to the same or related future stimuli. Therefore, the neurotrophic hypothesis in depression proposes that any impairment in the function of the brain-derived neurotrophic factor (BDNF) has an impact on the regulatory functions of this neurotrophic factor affecting the behavior and causing depression. In many studies, a decrease in the BDNF and BDNF pro-peptide level and their gene expression, as well as

BDNF receptor (Trk-B) were detected in the brain of patients with depression (Duman and Li, 2012; Pandey et al., 2010).

The Inflammation/Cytokine hypothesis: suggests that any disturbance in the immune system would have a great impact on changing the behavior associated with depression. Proinflammatory cytokines are therefore involved in the pathophysiology of depression through changing the white matter structure, brain global connectivity and functional activation, and increasing the level of pro-inflammatory biomarkers. Indeed, Tumor necrosis factor-alpha (TNF-α), interleukins (IL-1, IL-6), and C-reactive protein (CRP) levels are reported to increase in patients with depression (Lee and Giuliani, 2019). Microglia, which regulates inflammation, have also a contribution in the development of many neurodegenerative and psychological disorders including depression (Deng et al., 2020; Klawonn et al., 2021).

The Circadian Hypothesis: proposes that circadian rhythm disturbances resulting from stressful events and gene expression dysregulation are linked with mood disorders, especially abnormalities in the sleep/wake cycle are observed in patients with depression. Many studies showed the interconnection between sleep deprivation and the increment of pro-inflammatory cytokines such as IL-6, TNF-α, and CRP in the blood (Felger and Lotrich, 2013), between the circadian cycle and melatonin concentration (Zaki et al., 2018), the expression of the circadian genes in DA neurons of the ventral tegmental area (VTA), and the abnormality in the reward system (Chaudhury et al., 2015). In addition, a connection with the dysfunction in the hypothalamus (HT), monoamine projections, GABA, histamine, and serotonin, and the disturbance of the sleep/wake cycle has been linked with the development of the depressive disorder (Filatova et al., 2021) .

The GABA-glutamate-mediated hypothesis: GABA, the principal inhibitory neurotransmitter, has a critical role in the brain control of stress, and GABAergic transmission has an important role in the control of hippocampal neurogenesis and neural maturation. Alterations in the GABAergic system, such as reduction of GABA level in the plasma and the CSF, as well as the involvement of genetic polymorphisms in the GABA-A receptor subunit genes has been observed in patients with depression (Luscher

et al., 2011). Thus, there is a link between the GABA and/or glutamate neurotransmitter malfunction and depression, which leads to producing a loss of cognitive ability, accumulation of the extracellular glutamate, and cytotoxic damage to the neurons and glia. Additionally, glutamate malfunction leads to dysregulation of the growth factors and contributes to cell apoptosis (Sharpley, 2009).

The Cholinergic system: is involved in the pathogenesis of depression because the cholinergic system is connected with the hippocampus and the VTA and participate in the reward system functionality. Thus, any impairment will affect the reward system, which affects mood regulations. Additionally, imbalance in the central cholinergic neurotransmitter activity can contribute to depression (Jeon et al., 2015; Picciotto et al., 2012). Acetylcholine plays a major role in the sensory and emotional process, and any overactivity will be overrepresented with the emotions and mood of the patient and cause depression (Drevets et al., 2008).

1.5. Diagnosis of depression

Rating scales are used to identify the presence/absence and the severity of some psychiatric disorders, such as depression and anxiety. Additionally, these scales provide the clinician with information that helps in planning for appropriate treatment and tracking patients' progressions. They are presented in the form of Questionnaires using absolute values and are distinctively different from one scale to another, and used to avoid the struggle in detecting the efficacy of antidepressant agents (Smarr and Keefer, 2011).

Specific rating scales are used for each disorder, and the gold standard rating scales used for depression are the Hamilton Rating Scale for Depression (HAM-D or HRSD), The Beck Depression Inventory (BDI), and the Inventory of Depressive Symptomatology (IDS or QIDS). These scales are used in clinical trials in depression (Cusin et al., 2010). HAM-D is used to assess the severity of depression among patients (Worboys, 2013). BDI is used to assess efficacy of the psychoanalytically oriented psychotherapy in depressed patients, and IDS to detect the symptoms of depression according to the DSM criteria (Cusin et al., 2010) .

Other scales are also there, such as the Montgomery and Asberg Depression Rating Scale (MADRS) and The Zung Self-Report Depression Scale (Zung SDS). MADRS is used to measure depression severity, and to sensitize the effects of antidepressants, primarily tricyclic antidepressants (TCAs). Zung SDS is used to assess the symptoms in a broad-spectrum way by including the psychological, affective, cognitive, behavioral, and somatic aspects of depression. Some specific scales are also used, such as the Geriatric Depression Scale (GDS), which is used for elderly patients having depression with other medical illnesses (Cusin et al., 2010; Quilty et al., 2013; Zung, 1965).

1.6. Management of depression

There are two main methods used for the treatment of depression: pharmacological therapy and psychotherapy (Otte et al., 2016).

1.6.1. Pharmacological therapy

The available antidepressants are effective but differ in their effect on the brain chemistry (Mondimore, 2006), and mostly support the monoamine hypothesis (Ritter et al., 2019). These antidepressant agents act on the basis of nine different pharmacological mechanisms; two are classical while the others are new. The two classical or the first-generation antidepressants include the Monoamine oxidase inhibitors (MAOI) and TCAs (Lopez-Munoz and Alamo, 2009). The new antidepressants including the serotonin selective reuptake inhibitors (SSRI), the dual serotonin and norepinephrine reuptake inhibitor (SNRI), serotonin-2 antagonist/reuptake inhibitors (SARIs), norepinephrine and dopamine reuptake inhibitor (NDRI), noradrenergic and specific serotonergic antidepressants (NaSSAs), noradrenalin specific reuptake inhibitor (NRI) and serotonin reuptake enhancer were the second generation and some were novel drugs (Stahl, 1998; Yildiz, A. et al., 2002).

MAOIs act by blocking the metabolism of the monoamine neurotransmitters (5-HT, NE and DA), hence increase their availabilities. The MAOIs, include the classical MAOIs-Irreversible and nonselective (Phenelzine, Tranycylpormine, and Isocaronoxazid); Reversible inhibitor of MAO-A (RIMA) (Moclobemide), and Selective Inhibitor of

MAO-B (Deprenyl) (Stahl, 1998). TCAs act by a nonselective inhibition of the reuptake of 5-HT, NE, and DA into presynaptic storage vesicle in the brain (Ferguson, 2001), and differ in the degree and selectivity of inhibition of 5-HT and NE transporters. TCAs are different drugs in one group including serotonin reuptake inhibitor (SRI), norepinephrine reuptake inhibitor (NRI), anticholinergic-antimuscarinic drugs (M1), α1-adrenergic antagonist and anti-histamine (H1). They include Amitriptyline, Imipramine, Trimipramine, Clomipramine, Desipramine, Nortriptyline, Protriptyline, Doxepin, Amoxepine, and Maprotiline (Gillman, 2007; Stahl, 1998). SSRIs selectively inhibit serotonin transport causing a sudden increase in serotonin in the somatodendritic area of serotonergic neurons, and include Fluoxetine, Sertraline, Paroxetine, Fluvoxamine, and Citalopram (Yildiz, A. et al., 2002). SSRIs are the most widely prescribed antidepressant because of their safety and tolerability among the first line antidepressants (Thase, 2009). SNRIs are a dual action antidepressants with a non-selectivity for 5-HT and NE uptake and include Venlafaxine, which is a dose dependent drug, at low dose it acts as a SSRI, at medium to high acts as SSRI and NERI, and at high to very high dose, it acts as a DA reuptake inhibitor (Petersen et al., 2006; Ritter et al., 2019; Yildiz, A. et al., 2002). SARIs act by inhibiting serotonin reuptake and block serotonin 5-HT2A and 5-HT2C, including Nefazodone, Trazodone (Haller et al., 2014). NDRIs are a novel antidepressants that act selectively on noradrenergic and dopaminergic system and ignore the serotonergic system, and include Bupropion (Friedman and Anderson, 2014; Petersen et al., 2006). NaSSAs, include Mirtazapine, which has a unique pharmacological profile and it is a potent antagonist of central α_2 adrenergic auto-receptors and heteroreceptors and antagonist of postsynaptic serotonin 5-HT2 and 5-HT3 receptors. also has a potent binding to the H1 histamine receptor (Petersen et al., 2006). Mianserine is another NaSSA with a dual action used to treat depression associated with anxiety and agitation (Dell'Osso et al., 2011). NRIs include Reboxetine, which is the first selective NE reuptake inhibitor, and improve the symptoms of depression, energy, interest concentration, agitation, helplessness and hopelessness (Yildiz, A. et al., 2002). Serotonin reuptake enhancers, this group has only one drug, tianeptine, a tricyclic compound of dibenzo-thiazepine, acts by increasing the presynaptic uptake of serotonin and has no effect on the postsynaptic serotonin. It also increases serotonin fiber density in the neocortical and limbic forebrain areas. However,

tianeptine has no affinity for the α_1-adrenergic, H1, and muscarinic receptors (De Simoni et al., 1992; Yildiz, A. et al., 2002; Zhou et al., 2006).

The above mentioned antidepressants have a variety of side effects such as dry mouth, nausea, diarrhea, constipation, drowsiness, insomnia, dizziness, and/or lightheadedness (Laban and Saadabadi, 2021) for MAOIs and are contraindicated in cardio and cerebrovascular disease, children, epilepsy, hepatic disease, hyperthyroidism, and pheochromocytoma (Friedman and Anderson, 2014; Yildiz, A. et al., 2002). TCAs have an anticholinergic effect, cardiovascular effect such as conduction disturbances, hypotension, and tachycardia, and weight gain (Frommer et al., 1987; Settle, 1998). TCAs are highly toxic and cause a potential lethal cardiac arrythmias and seizure (Friedman and Anderson, 2014; Petersen et al., 2006; Stahl, 1998). SSRIs are of minor side effects such as anxiety, gastrointestinal disturbance, sleep disturbance, and sexual dysfunction, which is common (Haller et al., 2014). SNRIs have the same efficacy without the toxic effect of TCAs, however increased risk of suicidality and aggression in children, adolescence and young adults were observed (Petersen et al., 2006; Ritter et al., 2019). Orthostatic hypotension and priapism are the most serious adverse effect with SARIs (Ciraulo et al., 2004; Haller et al., 2014). Anxiety, headache, and tremor were commonly seen with NDRI and it is contraindicated in patients with seizures (Ciraulo et al., 2004; Friedman and Anderson, 2014; Petersen et al., 2006). Sedation and weight gain are some side effects with the use of NaSSAs (Petersen et al., 2006). Tianeptine, has some rare side effects such as abdominal pain, anorexia, asthenia, drowsiness, dry mouth, flatulence, gastralgia, insomnia, nausea, nightmares, vomiting and tachycardia (De Simoni et al., 1992; Zhou et al., 2006).

Moreover, some medications that are used to treat other diseases are demonstrated to be of use in depression such as thyroid medications, for depressed patients with underactive thyroid gland, lithium for manic-depressive disorder, psychostimulants that boost energy levels, concentration and mood such as Amphetamine. But it fell out of favor because of its tendency to raise blood pressure, causing symptoms of paranoia and suicidal depression when stopped. However, it is still used as adjuncts to other treatment like TRD and in depression associated with cancer and AIDS. Antipsychotic medications such as

sedative/hypnotics used to relief the sleeplessness symptoms of depression, and tranquilizers for anxiety associated with depression (Mondimore, 2006). Additionally, some humanized antibody treatments such as Tocilizumab (humanized anti-IL-6 receptor antibody), which was approved for Castleman's disease and arthritis is also used to treat depression. Usteknumab, an antibody against interleukin 12/23, decreases the symptoms of depression and anxiety in patient treated for psoriasis. Also, Ketamine, a novel antidepressant with a fast-acting effects (Hodes et al., 2015) is approved by the FDA for the treatment of resistant depression, PTSD, and heroin addiction (Zağlı et al., 2021).

Novel therapeutic agents can also be used to improve treatment of depression including triple monoamine reuptake inhibitors, atypical antipsychotic augmentation, dopamine receptors antagonists, corticotropin-releasing factor-1 receptor antagonist, glucocorticoid receptor antagonists, substance P receptor antagonists, N-methyl-D-aspartate receptor antagonists, nemifitide, omega-3 fatty acids, and melatonin receptor agonists (Holtzheimer and Nemeroff, 2008).

1.6.2. Psychotherapy

Psychotherapy is the second effective method used for treatment of depression and includes cognitive–behavioral therapy, behavioral activation therapy, psychodynamic therapy, problem-solving therapy, interpersonal therapy, and mindfulness-based therapy. This method produces effects that are equivalent to the previously mentioned pharmacotherapy. Cognitive-behavioral therapy shows how to identify negative, sorted thinking patterns that contribute to depression. Behavioral activation therapy increases the patient's positive activity to regain the pleasure feeling lost during depression. Psychodynamic therapy helps to explore and gain the nonsense emotions and thought to create a way of coping with changes. Problem-solving therapy allows to create and gain skills to identify, overcome and solve problems. Interpersonal therapy helps to identify and resolve relationships problems or any other social issues, and the mindfulness-based therapy allows patient to use meditation practices to clear their thought, feelings and experiences from negativity (Otte et al., 2016).

Other methods and techniques that are used for the management of depression are called somatic therapy and include electroconvulsive therapy (ECT), vagus nerve stimulation (VNS), deep brain stimulation (DBS), repetitive transcranial magnetic stimulation (rTMS), magnetic seizure therapy, transcranial direct current stimulation (tDCS). However, ECT is the best focal brain stimulation and most widely used non-pharmacological biological treatment for a variety of mental disorders such as TRD with a response rate of 50-70%, but has a cognitive side effect. VNS was approved by the FDA for the treatment of TRD and medication-resistant epilepsy and also used in combination with ECT. DBS was approved by the FDA to be used in severe obsessive-compulsive disorder and for the TRD. rTMS is used in the treatment of TRD and intractable seizure disorder. Magnetic seizure therapy is similarly used for depression but with fewer cognitive side effects as largely seen in ECT. tDCS is a non-invasive technique and similarly used in the treatment of depression (Al-Harbi, 2012; Holtzheimer and Nemeroff, 2008; Otte et al., 2016).

1.7. Medicinal plants used traditionally for depression

Medicinal plants are used traditionally for centuries as a sum of knowledge, skill, and practice by local practitioners and traditional healers to prevent, diagnose, maintain, and improve physical diseases and mental disorders (Mahomoodally, 2013). The leaves and roots are the main and most parts used. Furthermore, according to the WHO, about 3.5 billion people in developing countries rely on using these medicinal plants regularly (Moges and Moges, 2020). In Africa, there are about 45,000 species of plants and 5,000 species that have medical properties, and are used traditionally (Mahomoodally, 2013). Moreover, as a result of the high diversity of floral composition in Ethiopia, where there are almost 6500–7000 different species, out of which 800 species are of medicinal properties used to treat about 300 different diseases (Moges and Moges, 2020). More than 80% of Ethiopian people uses medicinal plants traditionally for protecting and promoting human physical and mental well-being due to effectiveness, safety and high medical values of bioactive components (Bishaw, 1991).

Medicinal plants are easily accessible and available to people in low-and-middle income communities and are known to be efficacious. Therefore, inadequate accessibility, affordability, and lack of effective modern pharmaceutical medicinal treatment were the main reason for using traditional medicine regularly by almost 80% of the world population (Ekor, 2014). This is evidenced by the global market of these traditional medicines is estimated to be $ 83 billion annually according to the WHO (Wassie et al., 2015).

Plant-derived natural medicines have a crucial role in the development of natural drugs, which have antidepressant activity and are used for depression management. Many plants that are used in depression treatment act by normalizing the disturbed neurotransmitters in the brain or related mechanisms, including the 5-HT system (hypericum species), NE system (*Valeriana wallichii*), the DA system (*Rosmarinus officinalis*), GABAergic system (*Asparagus racemosus*), Glutamate system (*Siphocampylus verticillatus*), neurotrophins (*Polygala tenuifolia*), HPA axis (*Ptychopetalum olacoides*) and MAO (*Paeonia lactiflora*) (Farahani et al., 2015).

1.8. The experimental plant

1.8.1. The genus *Carissa*

Carissa is a genus that belongs to the family Apocynaceae, which has about 250 genera, and 2000 species. The genera are classified under two subfamilies namely Plumieroideae, and Apocynoideae. The genus *Carissa* belongs to the Plumieroideae sub family (Evans, 2009), and it has about 36 species, which are highly distributed in the tropical and subtropical regions of Africa, Asia, Australia, and Oceania, and cultivated as ornamental plants in America. *Carissa* as an evergreen shrub is about 2–10 m tall and it possesses glossy foliage and fragrant, starry-white, jasmine-like flowers, and the fruits are ornamental and edible, scarlet to crimson in color, oval in shape, and are produced after flowering". All parts of *Carissa* plants, the stems, root barks, leaves, fruits, and seeds are used in food industries, as normal diet in some tribes and as a traditional medicine for the treatment of several illnesses such as headache, chest complaints, rheumatism, edema, gonorrhea, syphilis, rabies and as diuretics (Kaunda and Zhang, 2017; Patel, 2013).

1.8.2. *Carissa spinarum* Linn.

Carissa spinarum Linn. (Apocynaceae) is a medicinal species known as the magical herb in most African countries. Sometimes it is called *Carissa edulis Vahl*, and all parts of this magical herb, the roots, the leaves, and even the fruits (Fig.1) are used in the treatment of several diseases due to its high contents of bioactive secondary metabolites, such as alkaloids, cardiac glycosides, saponins, flavonoids, tannins, anthraquinones, total phenols, terpenoids (Ansari and Patil, 2018; Nantongo et al., 2018; Teke and Kuete, 2014). *C. spinarum* has different vernacular names, Hagmsa (in Affan Oromo), and Agam (in Amharic). It is extensively used in Ethiopian folklore medicine. The roots are used before delivery to shorten labor as well as, for wounds, (Moges and Moges, 2020). Roots mixed with some plants are used for Evil eye (Gidey and Asfaw, 2015; Moges and Moges, 2020; Teklehaymanot, 2009), and in combination with *Withania somnifera* it is used to treat depression (Moravec et al., 2014). The mixed powdered roots with water are used as a snake repellent; fresh grounded roots are used for external injuries

in cattle, also for swelling of throat/sore throat, stomach-ache, muscle cramps, malaria, ascariasis and mental disorder (Teklehaymanot, 2009). In southern Ethiopia, roots are boiled for treating diarrhea (Tefera and Kim, 2019) as well as, for the treatment of cancer (Esubalew et al., 2017). In Nigeria, it is used for the treatment of epilepsy, headache, syphilis, rheumatism and sickle cell anemia (Ibrahim et al., 2016).

Several of the traditional uses of *Carissa spinarum* were supported scientifically by several pharmacological studies including diuretic (Kebamo et al., 2015; Nedi et al., 2004), antipyretic (Gitahi, 2015), antinociceptive activity (Maina et al., 2015; Ngulde et al., 2013a) anticonvulsant (Ya'u et al., 2015, 2008), anti-inflammatory and antioxidant (Woode et al., 2007), analgesic and anti-inflammatory (Hassan et al., 2010; Ibrahim et al., 2007; J. Ya'u et al., 2017), antimicrobial (Berhanu et al., 2020; Jenson, 2018; Ngulde et al., 2013b), antiviral (Tolo et al., 2010), hepatoprotective and antioxidant (Hegde and Joshi, 2010), anti-plasmodial (Oduor et al., 2019), hypoglycemic (El-Fiky et al., 1996), and antioxidant (Jagadeeshwar Rao et al., 2005) activity.

The root bark extract of the plant is also reported to possess antidepressant activity (Ya'u et al., 2017). Therefore, the aim of this study is to evaluate the antidepressant-like activity of the solvent fractions of the root bark of *C. spinarum* Linn, in rodents.

Fig 1: Photograph of *Carissa spinarum* Linn. a, Leaves; b, Flowers; c, Fruit; d, Roots

(Photograph taken by Hana Saif – Aug, 2020)

1.9. Rationale for the study

Depression is shown to be one of the two most disabling mental disorders and a leading cause of burden worldwide (Santomauro et al., 2021). Although there are several highly effective drugs that are used for treatment of depression, almost all of them are associated with several side effects. First generation antidepressants are associated with dry mouth, dizziness, drowsiness, prolonged orgasms, decreased sexual function, constipation, inability to drive, headache, insomnia, sadness, and some show contradictive outcomes (Friedman and Anderson, 2014; Laban and Saadabadi, 2021). The second generations can cause sexual dysfunction (Yildiz, A. et al., 2002), orthostatic hypotension (Haller et al., 2014), and are contraindicated with some diseases. They also showed potential interactions with food and other medications, and were linked to hepatic impairment and increased risk of diabetes (Ashraf et al., 2021).

Moreover, about 50-60% of patients fail to receive antidepressant therapy and those on treatment can develop resistance during treatment. In order to overcome these side effects, low effectiveness, and inaccessibility (Lee and Bae, 2017; Otte et al., 2016), many scientists are seeking alternatives to conventional antidepressants from medicinal plants. A wide variety of phytochemical constituents of various medicinal plants such as polyphenols (flavonoids, phenolic acids, lignanes, coumarins), alkaloids, terpenes and terpenoids, saponins and sapogenins, amines, and carbohydrates, have been shown to possess antidepressant effect (Bahramsoltani et al., 2015; Malik et al., 2020). Their accessibility and affordability, increases the reason for using these medicinal plants in both developed and developing countries (Wassie et al., 2015).

Carissa spinarum Linn. (Apocynacea), a herb which is widely distributed in Africa, was used traditionally to treat various diseases, such as mental disorder including depression (Moravec et al., 2014; Teklehaymanot, 2009). Indeed, a previous study conducted elsewhere has demonstrated the antidepressant-like effect of the plant (Ya'u et al., 2017). In light of the previous work, the present study aimed to further investigate the antidepressant-like effect of the solvent fractions of the root bark of *C. spinarum* and their possible mechanistic action (s).

2. OBJECTIVES

General objective

- To investigate the antidepressant-like activity of the solvent fractions of the root bark of *Carissa spinarum* Linn. in rodents.

Specific objectives

- To determine the antidepressant-like activity of the solvent fractions of the root bark of *C. spinarum* using a tail suspension test in mice;
- To evaluate the antidepressant-like activity of the solvent fractions of the root bark of *C. spinarum* using a forced-swim test in rats;
- To determine the antidepressant-like activity of the solvent fractions of the root bark of *C. spinarum* using a muricidal behavioral test in rats;
- To rule out the false-positive antidepressant-like activity of the solvent fractions of the root bark of *C. spinarum* using an open field test;
- To quantify the changes caused in the levels of markers for depression such as serum corticosterone by the fractions using electrochemiluminescence immunoassay (ECLIA);
- To identify the possible mechanism (s) of action of the active fraction using various pharmacological agents;
- To quantify the total flavonoids, total phenols, and total alkaloids of the active fraction based on the preliminary analysis.

3. MATERIALS AND METHOD

3.1. Drugs, chemicals, and reagents

Cyproheptadine HCl (Algorithm S.A.L - Lebanon); Diazepam BP (Gland Pharma Limited - Denmark); Ketamine Hydrochloride USP (Rotex Medica - Germany); L-Arginine HCl (Vita Pharmaceutical - Syria); Prazosin HCl (Mylan Inst., RKFD - USA); Standard Imipramine HCL BP (Remedica - Cyprus); Sulpiride (Sanofi-Aventis - France); Yohimbine (Sun Naturals - USA); Atropine (BDH Chemicals - England); Absolute Ethanol 99.9% (Iso Lab Chemicals - India); Chloroform (Loba Chemie - India); Dimethyl sulfoxide (DMSO) (Riedel.de Haen - Germany); Ethyl Acetate (Lobe Chemie - India); Hydrochloric acid HCl (BDH Laboratory Supplies - England); Methanol (Sisco Research Laboratories - India); Methylene Blue (BDH Chemicals - England); n-Butanol (Loba Chemie - India); Normal saline Solution (Sansheng Pharmaceutics - Ethiopia); Petroleum Ether 40-60˚C (Loba Chemie - India); Tween 80 (Uni-Chem - India), Aluminum Chloride Hexahydrate (Loba Chemie - India); BCG (Sisco research laboratories - India); Citric acid (Avonchem - UK); Disodium hydrogen orthophosphate Na_2HPO_4 (BDH Chemicals - England); Follin Ciocalteu; Gallic acid (Merch - Germany); Potassium Acetate (Blulux laboratories - India); Quercetin dihydrate (Sigma Aldrich - Germany); Sodium carbonate (Loba Chemie - India); Sodium hydroxide pellets (Loba Chemie - India); Distilled water; all chemicals and reagents were obtained from the Department of Pharmacology and Clinical pharmacy, Pharmaceutical chemistry, and Pharmacognosy, AAU; All drugs were purchased from pharmacies in Ethiopia, Yemen, and USA, while the chemicals were purchased from their respective vendors in Ethiopia. All were of analytical grades.

3.2. Plant Material

The roots of *C. spinarum* was collected from Ashewa Meda, around Addis Ababa, Liyu zone, Burayu city administration, Oromia, Ethiopia, in August 2020. The identification and authentication of the plant sample was performed by Mr. Melaku Wondafrash, a taxonomist and a voucher specimen (HS #001) was deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University for future reference.

3.3. Experimental Animals

Healthy Swiss albino mice (6-8 weeks, weighing 20–30 g) and Sprague Dawley rats (8-12 weeks, 200-250 g) of either sex bred at the animal house of the School of Pharmacy, Addis Ababa University (AAU) were used. All animals were housed in groups of 6 animals per cage and had access to standard laboratory pellet and water *ad libitum* and maintained on 12/12 h of light/dark cycle at room temperature. The experimental animals were acclimatized to the laboratory conditions for 5 days before conducting the pharmacological studies (done in late afternoon, 4:00 pm). Moreover, all animals used were handled and cared based on the internationally accepted Guidelines for the Care and Use of Laboratory Animals (National Research Council, 2010). Ethical clearance was obtained from an Ethical review board of the School of Pharmacy, College of Health Sciences, AAU (ERB/SOP/176/12/2020).

3.4. Extraction Procedure

3.4.1. Crude Extract

The root of *C. spinarum* was cleaned and washed carefully from dirt and remaining soil using tap water. The root bark was then separated from the root wood and cut into small pieces, and allowed to dry in open air under shade. The dried root bark was ground into a fine powder using mortar and pestle. Five hundred fifty grams of the ground root bark was weighed using Kern analytical balance (ALJ 220-4 – the UK). It was then defatted by soaking in 1L of petroleum ether overnight, filtered, and allowed to dry on a

clean piece of Aluminum. After complete evaporation of the petroleum ether, the sample was macerated with 1.5 L of 70% of ethanol for 72 h with frequent shaking. The maceration process was repeated twice using the same volume. The extract was then filtered using a cotton gauze followed by Whatman filter paper No. 1, and filtration was accelerated using a vacuum pump V700 (Buchi Labortechnik - Switzerland). The filtrate was then concentrated under reduced pressure using a rotary evaporator at 40˚C (Heidolph Rotary Evaporator R 10-280 rpm - Germany) and stored in a refrigerator at 4˚C. A total of 50.72 g of a sticky dark brownish-colored crude extract was obtained with a percentage yield of 9.22%. The crude extract was then used for a pilot study to check whether the extract had effect as reported. Once that was ascertained, the remaining extract was used for fractionation.

3.4.2. Solvent Fractionations

Thirty-three grams of the extract was taken and mixed with acid-washed sand and applied into a small thimble for fractionation using different solvents in a Soxhlet apparatus (Pyrexquickfit - UK). The sequence of solvents used for fractionation was according to their polarity index (n-butanol, ethyl acetate, and water). The n-butanol and ethyl acetate fractions were then collected sequentially and dried under reduced pressure using a rotary evaporator at 40˚C. Finally, the marc left was macerated with 1 L of distilled water and then freeze-dried using a lyophilizer (Labconco, Console Freeze Dry system, USA) to get the aqueous fraction. The final sample obtained were weighed and the percent yields were calculated and found to be 21.00% for the n-butanol fraction (CSB), 11.55% for the ethyl acetate fraction (CSE), and 61.7% for the aqueous fraction (CSA). The CSB and CSE were sticky and light brown in color, while the CSA was black. The fractions were kept in tightly closed containers and stored in a refrigerator at -20˚C until used for the main experiment.

3.5. Grouping and dosing of animals

Five groups of animals of either sex each having 6 animals per group were randomly assigned for every model and each solvent fraction. Group I, served as a negative control group and received a vehicle (2% Tween 80), group II served as a positive

control and received the standard drug Imipramine (30 mg/kg), group III-V received increasing doses of the solvent fractions 50, 100 and 200 mg/kg, respectively. The solvent fractions, and the standard drug were dissolved in 2% Tween 80 and prepared and administered orally one hour before the experimental sessions. The maximum volume administered was 10 ml/kg (Diehl et al., 2001). Selection of the dose of the standard drug was based on previous studies (Porsolt et al., 1977), while doses of the solvent fractions were based on pilot studies conducted before the main experiment.

3.6. Antidepressant activity tests

3.6.1. Tail suspension test

Tail suspension test (TST) was performed as described elsewhere (Steru et al., 1985). Following 1 h after administration of the vehicle/standard/fractions, each mouse was hung in an upside-down position by its tail using adhesive tape, applied 1 cm from the tip of the tail, to a countertop of 35-50 cm above the floor. The test was recorded using a digital video camera (Sony video camera DSC-W120 - Japan), and the duration of immobility was scored and calculated for the entire duration of the test (6 min) in seconds using a stopwatch software, the XNote timer (Can et al., 2012). The duration of immobility is defined as the absence of any movement of the head and the body except for those necessary for respiration (Can et al., 2012; Costa-Nunes et al., 2015).

3.6.2. Forced swim test

The forced swim test (FST) or the Porsolt behavioral despair test in rats was performed as described in (Porsolt et al., 1978) with some modifications. There are two sessions in the FST. The initial 15 min pretest session followed by a 5 min test session 24 h later. The rats were forced to swim in an inescapable situation in a transparent glass cylinder (40 cm in height; 18 cm in diameter) filled with water of 20 cm depth, where their hand paws can't touch the bottom. The solvent fractions in this study were administered 24, 4, and 1 h before the test. Each time the rat was removed, it was dried using a clean towel to maintain the body temperature and avoid hypothermia, and returned to the cage. Similarly, water was changed and replaced. Only the test sessions were video

recorded using a digital video camera, which was then used to score the duration of immobility in seconds (Detke et al., 1997, 1995). The duration of immobility was scored using a stopwatch software, the XNote timer (Can et al., 2012). Floating and absence of any struggling behavior except those necessary for keeping the head above water for respiration were taken as an immobile behavior, which implies the development of an adaptive response to the stressful situation in water (Krishnan and Nestler, 2011).

3.6.3. Open field test

Open field test (OFT) measures the general locomotor activity and rule out any false-positive effect of the solvent fraction on mice. The OFT apparatus is a rectangular inescapable box (68 cm x 68 cm x 45 cm) with a surface marked horizontally and vertically with lines forming a grid of 16 squares and a 60 W lamp is attached above the box (Brenes Sáenz et al., 2006; Demissie et al., 2017; Fekadu et al., 2016; Gould et al., 2009). Solvent fractions were administered orally one hour before the test. The mouse was then placed in the middle and crossing the central and the peripheral squares were video tracked and counted for 5 min. The inner surface of the box was swapped and cleaned using alcohol and cotton to avoid any olfactory cues after each animal (Demissie et al., 2017; Gould et al., 2009).

3.6.4. Muricidal behavioral test

Sprague Dawley rats (300-350g) were isolated in individual cages for a period of 5-6 weeks with access to food and water *ad libitum*. Muricidal behavior was determined after removing food for 3-4 h and then presenting one mouse into each of their cages. A rat that bit the mouse on the neck of the cervical spinal cord and killed it within seconds was chosen for the test and called a killer rat. The chosen killer rats were taken individually and orally administered as mentioned under grouping and dosing section. A mouse was presented to each cage 30, 60, and 120 min following administration and the mouse was removed 15–45 sec after the attack to avoid being eaten up. This procedure was tracked by video recording for five minutes. Failure of attacking or killing behavior

within the 5 min was considered as inhibition of muricidal behavior and latency of attack was measured (Horovitz et al., 1965; McMillen et al., 1988; Valzelli et al., 1981).

3.7. Serum Corticosterone Assay

After completing the FST and the TST, each animal was immediately anesthetized using Ketamine (80 mg/kg, i.p) and Diazepam (10 mg/kg, i.p) (Wixson and Smiler, 1997) and a cardiac puncture was performed. Blood was collected in an SST tube and kept at room temperature for 60 min. It was then centrifuged at 1000 g for 10 min at 4˚C and the separated serum was stored at -80˚C until assayed (Kim et al., 2018). Corticosterone in rodents is a glucocorticoid hormone which is secreted from the adrenal cortex in different stressful conditions such as FST and TST. Thus, it was analyzed and measured using ECLIA according to the manufacturer's instructions (Cobas e 41-Roche Diagnostics GmbH, Mannheim, Germany).

3.8. Evaluation of Possible Mechanisms of Action

According to the pathophysiology of depression and the mechanism of action of conventional antidepressants, possible mechanism (s) of action of the active solvent fraction of the root bark of *C. spinarum* (CSE) was investigated by assessing the involvement of noradrenergic, serotonergic, dopaminergic, nitric oxide, and cholinergic-muscarinic system using different drugs at doses that do not modify the locomotor behaviors of rats in FST (Abiola et al., 2019; Akinpelu et al., 2017; Mensah, 2016). Rats were divided randomly into nine different groups, each comprising of 6 rats. All drugs and the extract were dissolved in 0.5% v/v DMSO in normal saline. Prazosin (1 mg/kg, i.p., an α1-adrenoceptor antagonist) and yohimbine (1 mg/kg, i.p., an α2-adrenoceptor antagonist) used for the noradrenergic system; cyproheptadine (3 mg/kg, i.p., a 5-HT_2 receptor antagonist) for the serotonergic system; sulpiride (50 mg/kg, i.p., a dopamine D2 receptor antagonist) for the dopamine system; atropine (1 mg/kg, i.p., a muscarinic cholinergic receptor antagonist) for the cholinergic system; and L-Arginine (750 mg/kg, i.p., a precursor of NO) and methylene blue (10 mg/kg, i.p., an inhibitor of soluble guanylate cyclase (sGC)) used for the NO system. All drugs were administered 15 min

before administration of CSE (50 mg/kg, p.o.) and the rats were subjected to FST 60 min after administration of the fraction.

3.9.Quantification of phytochemical constituents

3.9.1. Total flavonoids content

The total flavonoid content (TFC) of the solvent fractions was determined by Aluminum chloride colorimetric method as described elsewhere (Gandagule et al., 2018) with minor modification. Using quercetin (1mg/ml) as a standard solution, a series of concentrations (25, 12.5, 6.25, 3.125, and 1.5625 µg/ml) were prepared in methanol. 10 mg of each solvent fraction was dissolved in 10 ml of methanol to get 1mg/ml of the stock solution. 0.5 ml of each solvent fraction and 1 ml of the standard solution was mixed with 100 µl of 10% aluminum chloride, 100 µl of 1M potassium acetate and 2.8 ml of distilled water in a test tube. The mixture of each were incubated for 30 minutes at room temperature. The absorbance was measured at 415 nm using a UV-spectrophotometer (Jenway 6500 - England). The same procedure was followed to prepare the blank solution. A linear calibration curve was plotted. TFC of each solvent fraction was obtained using the formula below and expressed as milligram of quercetin equivalent per gram (mg QE/g) of the solvent fractions.

$$\mathbf{TFC} = \frac{C * V}{M * 1000}$$

Where C is the sample concentration of each solvent fraction (µg/ml) from the calibration curve, V is the volume of methanol used to dissolved each solvent fraction and M is the mass of the dry solvent fraction used in gram. The assay was conducted in triplicates and the average value was recoded.

3.9.2. Total phenols content

The total phenol content (TPC) of the solvent fractions was determined using Follin-Ciocalteu method as described elsewhere (Maria et al., 2018) with minor modification. Follin-Ciocalteu reagent (2N) was diluted (1:20) with distilled water and used. Using a standard solution of gallic acid (50 µg/ml), a series of concentration ((25,

12.5, 6.25, and 3.125 µg/ml) were prepared. 1 ml of each fraction solvents (250µg/ml) and 1 ml of the standard solution, each was mixed with 0.5 ml of Follin-Ciocalteu reagent in a test tube and allowed to stand at room temperature for 8 min. Sodium carbonate (2 ml of 7.5%) solution was added and the mixture was allowed to stand for 30 minutes at ambient temperature. The absorbance was measured at 765 nm using a UV-spectrophotometer. The same procedure was followed to prepare the blank solution. A linear calibration curve was plotted. TPC was read from the curve and expressed as milligram of gallic acid equivalent per gram (mgGAE/g) of the solvent fractions. The assay was conducted in triplicates and the average value was recoded.

3.9.3. Total alkaloids content

The total alkaloid content (TAC) of the solvent fractions was determined using Bromocresol green solution (BCG) and phosphate buffer solution, which were prepared as described elsewhere (Van Tan, 2018). Using a standard pure atropine dissolved in methanol (1mg/ml), a series of concentrations (0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/ml) were prepared. 2 ml of each solvent fraction (1 mg/ml) was dissolved in 2N HCl and then filtered using Whatman filter paper no.1. Then 1ml of the filtered solution was used and transferred to a separating funnel and washed twice with 5 ml of chloroform. The pH was neutralized using 0.1 N NaOH. Then, 5 ml of both BCG solution and phosphate buffer were added and shaken vigorously. Then 5ml of chloroform was added and the formed complex was transferred to a 10 ml volumetric flask and diluted to the volume with chloroform. The absorbance of the formed complex was measured at 470 nm. A blank and a standard solution were prepared using the same procedure without adding atropine (Tabasum et al., 2016). A linear calibration curve was plotted. TAC was expressed as milligram of Atropine equivalent per gram (mgATE/g) of the solvent fractions. The assay was conducted in triplicates and the average value was recoded.

3.10. Statistical analysis

The results obtained from the experiments were analyzed using a Statistical Package for the Social sciences (SPSS) windows version 25 using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The results were expressed as mean ± standard error of mean (SEM) and the level of significance was set at *P*-value of less than 0.05. The curves for the quantification of the phytochemical constituents was constructed using Graph Pad Prism software version 8.00 for windows (Graph Pad Software Inc., San Diego, California, USA).

4. RESULTS

4.1.Tail suspension test

Data obtained for the three fractions in the TST model are presented in Table 1 & 2. Mice treated with the three doses of CSE produced a significant reduction ($p< 0.001$) in the duration of immobility compared to controls. Duration of immobility appeared to decrease with dose, whereby the middle (100 mg/kg, CSE100) and the higher (200 mg/kg, CSE200) doses produced a significantly higher reduction ($p < 0.05$) than the lower dose (50 mg/kg, CSE50). CSB also produced the same pattern as that of CSE, except that CSB200 had a comparable effect to that of IP30. Indeed, the reduction in duration of immobility produced by the standard (IP30) was significantly greater ($p<0.05$) than those produced by all doses of CSE as well as CSB50 and CSB100. By contrast, CSA did not produce any appreciable reduction in the duration of immobility at the doses employed in the present study. When comparison was done between CSE and CSB it was revealed that no apparent difference was observed among the various doses of the two fractions, except CSB200 ($p<0.001$) produced a significantly greater reduction than CSE50.

Table 1: Antidepressant-like effect of ethyl acetate and n-butanol fractions of the root bark of *C. spinarum* in mice using tail suspension test

Treatment	Dose (mg/kg)	Duration of Immobility (seconds)	% Reduction
Control	10 ml/kg	218.10 ± 4.36	-
Standard (IP)	30	77.40 ± 10.25^{a3}	64.41%
	50	152.60 ± 3.21^{a3b3d2e2}	30.03%
CSE	100	110.30 ± 2.84^{a3b1c2}	46.43%
	200	108.40 ± 8.16^{a3b1c2}	50.30%
	50	157.30 ± 4.88^{a3b3d1e3}	27.88%
CSB	100	122.60 ± 8.82^{a3b1c1}	43.79%
	200	96.40 ± 10.10^{a3c3}	55.80%

Values are presented as mean ± SEM and analyzed using a One-way ANOVA followed by Tukey's Post hoc test; n = 6; Control received 2%Tween 80; IP, imipramine; CSE, *Carissa spinarum* Linn. ethyl acetate fraction; CSB, *Carissa spinarum* Linn. n-butanol fraction; $^{1:}$ $p < 0.05$; $^{2:}$ $p < 0.01$; $^{3:}$ $p < 0.001$; a, compared to control; b, compared to imipramine; c, compared to 50 mg/kg; d, compared to 100 mg/kg; e, compared to 200 mg/kg.

Table 2: Antidepressant-like effect of an aqueous fraction of the root bark of *C. spinarum* in mice using tail suspension test

Treatment	Dose (mg/kg)	Duration of Immobility (seconds)	% Reduction
Control	10 ml/kg	188.70 ± 8.59	-
Standard (IP)	30	111.90 ± 2.98[a2]	40.70%
	50	136.90 ± 10.57	27.45%
CSA	100	192.30 ± 25.94[b2]	-1.91%
	200	164.10 ± 13.01	13.04%

Values are presented as mean ± SEM and analyzed using a One-way ANOVA followed by Tukey's Post hoc test; n = 6; Control received 2%Tween 80; IP, imipramine; CSA, *Carissa spinarum* Linn aqueous fraction [1:] $p < 0.05$; [2:] $p < 0.01$; [3:] $p < 0.001$; a, compared to control; b, compared to imipramine; c, compared to 50 mg/kg; d, compared to 100 mg/kg; e, compared to 200 mg/kg.

4.2. Forced swim test

As the CSA was devoid of effect in the TST, FST was carried out for the CSE and CSB fractions. Table 3 summarizes data obtained from the FST using the two fractions with different doses used in the study. Accordingly, both fractions significantly reduced ($p<0.001$) duration of immobility in a dose-dependent manner compared to controls. In addition, the lower dose of both fractions had a significantly lower ($p<0.01$) reduction than the middle and higher doses, though no detectable difference was noted between the latter two doses. Moreover, CSE50 and CSB50 produced a significantly lower reduction ($p<0.001$) in duration of immobility than IP30. However, though CSB100 and CSB200 also produced a significantly lower ($p<0.001$) duration of immobility than IP30, no apparent difference was observed between IP30 and the other two doses of CSE (CSE100 & CSE200). Unlike TST, in FST, CSE tended to produce a consistently higher effect with each dose employed than the corresponding doses of the CSB. For instance, CSE200 exhibited a significantly greater reduction in immobility time than CSB100 ($p<0.01$) and CSB200 ($p<0.05$).

Table 3: Antidepressant-like effect of ethyl acetate and n-butanol fractions of the root bark of *C. spinarum* in rats using forced swim test

Treatment	Dose (mg/kg)	Duration of Immobility (seconds)	% Reduction
Control	10 ml/kg	190.46 ± 1.73	-
Standard (IP)	30	67.25 ± 3.60^{a3}	61.59%
	50	132.70 ± 8.28^{a3b3d3e3}	32.84%
CSE	100	85.10 ± 3.85^{a3c3}	56.93%
	200	78.30 ± 3.03^{a3c3}	60.37%
	50	136.80 ± 6.62^{a3b3d3e3}	28.17%
CSB	100	103.80 ± 2.66^{a3b3c2}	45.50%
	200	100.60 ± 3.65^{a3b3c3}	47.18%

Values are presented as mean ± SEM and analyzed using a One-way ANOVA followed by Tukey's Post hoc test; n = 6; Control received 2%Tween 80; IP, imipramine; CSE, *Carissa spinarum* Linn. ethyl acetate fraction; CSB, *Carissa spinarum* Linn. n-butanol fraction; [1:] $p < 0.05$; [2:] $p < 0.01$; [3:] $p < 0.001$; a, compared to control; b, compared to imipramine; c, compared to 50mg/kg; d, compared to 100mg/kg; e, compared to 200mg/kg.

4.3. Open field test

Table 4 depicts summary of the data obtained from the OFT. Accordingly, neither the standard nor the fractions produced a significantly different number of crossings compared to controls. Similarly, no apparent difference was noted between the different doses of the fractions and the standard, ruling out the possibility that the antidepressant like was not due to non-specific psycho-stimulation.

Table 4: The effect of ethyl acetate and n-butanol fractions of the root bark of *C. spinarum* on locomotion in mice using open field test.

Treatment	Dose (mg/kg)	Number of squares crossed		
		Peripheral	Central	Total
Control	10 ml/kg	80.00 ± 5.51	6.83 ± 2.10	86.83 ± 7.06
Standard (IP)	30	77.50 ± 9.58	7.50 ± 1.36	85.00 ± 9.52
	50	73.67 ± 17.30	7.33 ± 1.65	81.00 ± 18.02
CSE	100	68.50 ± 11.29	7.67 ± 1.84	76.17 ± 12.48
	200	82.33 ± 12.81	10.67 ± 4.23	93.00 ± 15.78
	50	69.00 ± 15.01	5.67 ± 2.59	74.67 ± 16.49
CSB	100	65.67 ± 16.49	5.83 ± 1.14	71.50 ± 16.18
	200	62.00 ± 21.88	4.67 ± 2.35	66.67 ± 23.72

Values are presented as mean ± SEM and analyzed using a One-way ANOVA followed by Tukey's Post hoc test; n = 6; Control received 2%Tween 80; IP, imipramine; CSE, *Carissa spinarum* Linn. ethyl acetate fraction; CSB, C*arissa* s*pinarum* Linn. n-butanol fraction

4.4. Muricidal behavioral test

As described in Table 5, control rats displayed muricidal behavior throughout the observation time. Treatment with the standard and fractions reduced this behavior, as the number of animals exhibiting this behavior decreased with time and dose. Whilst IP30 treated rats did not attack the mouse at all during the observation period, the fractions were able to significantly prolong the latency to attack. Latency was better prolonged with CSE than CSB. This is clearly seen 30 min following administration, although inconsistencies were observed in the other time points.

Table 5: Anti-muricidal effect of ethyl acetate and n-butanol fractions of the root bark of *C. spinarum* in rats

Treatment	Dose (mg/kg)	Number of animals with muricidal behavior			Median Latency of Attack in seconds		
		30 min	60 min	120 min	30 min	60 min	120 min
Control	10 ml/kg	6/6	6/6	6/6	0.60	0.60	0.60
Standard (IP)	30	0/6	0/6	0/6	-	-	-
	50	4/6	2/6	2/6	105.90	21.60	140.40
CSE	100	3/6	1/6	1/6	244.20	240.00	139.80
	200	2/6	2/6	0/6	197.10	80.10	-
	50	3/6	2/6	0/6	87.60	72.00	-
CSB	100	2/6	2/6	2/6	12.60	0.60	157.50
	200	2/6	2/6	1/6	8.10	10.80	0.60

Values are presented as median; n= 6; Control received 2%Tween 80; IP, imipramine; CSE, *Carissa spinarum* Linn. ethyl acetate fraction; CSB, *Carissa spinarum* Linn. n-butanol fraction; [1:] $p < 0.05$; [2:] $p < 0.01$; [3:] $p < 0.001$; a, compared to control; b, compared to imipramine; c, compared to 50 mg/kg; d, compared to 100 mg/kg; e, compared to 200 mg/kg

4.5. Serum Corticosterone Assay

The effect of CSE and CSB on serum corticosterone level in TST is shown in Table 6. CSE at all doses significantly ($p < 0.01$) reduced the serum corticosterone level in a dose dependent manner compared to controls. Although IP30 significantly lowered ($p<0.05$) the level compared to CSE50, no apparent difference was observed between IP30 and the other two doses. All doses of CSB did not have a significant effect on corticosterone level, as no detectable changes were observed compared to controls. Moreover, except with CSB50 ($p<0.05$), no significant difference was observed with the other doses when compared to IP30.

Table 6: The effect of ethyl acetate and n-butanol fractions of the root bark of *C. spinarum* on serum corticosterone level in mice using tail suspension test

Treatment	Dose (mg/kg)	Serum Corticosterone Level (nmol/l)	% Reduction
Control	10 ml/kg	63.72 ± 7.80	-
Standard (IP)	30	19.57 ± 2.65[a3]	69.29%
CSE	50	39.18 ± 3.91[a2b1]	38.51%
	100	31.11 ± 2.62[a2]	51.27%
	200	27.09 ± 3.96[a3]	57.49%
CSB	50	50.59 ± 8.48[b1]	20.61%
	100	44.96 ± 11.57	29.44%
	200	34.35 ± 7.26	46.09%

Values are presented as mean ± SEM and analyzed using a One-way ANOVA followed by Tukey's Post hoc test; n = 5; Control received 2%Tween 80; IP, imipramine; CSE, *Carissa spinarum* Linn. ethyl acetate fraction; CSB, *Carissa spinarum* Linn. n-butanol fraction; [1:] $p < 0.05$; [2:] $p < 0.01$; [3:] $p < 0.001$; a, compared to control; b, compared to imipramine.

Serum corticosterone level was also measured in rats subjected to FST. As demonstrated in Table 7, CSE50, CSB50 and CSB100 failed to affect corticosterone level. By contrast, CSE100 ($p<0.05$), CSE200 ($p<0.05$) significantly reduced compared to controls. On the other hand, IP30 was found to have a significantly greater reduction ($p<0.05$) compared to all doses of CSE. However, greater individual variation precluded statistically significant between standard and the CSB fraction.

Table 7: The effect of ethyl acetate and n-butanol fractions of the root bark of *C. spinarum* on the serum corticosterone level in rat using forced swim test

Treatment	Dose (mg/kg)	Serum Corticosterone Level (nmol/l)	% Reduction
Control	10 ml/kg	72.66 ± 5.14	-
Standard (IP)	30	28.13 ± 3.86[a3]	61.29%
	50	59.92 ± 4.38[b3]	17.53%
CSE	100	53.08 ± 1.53[a1b2]	26.95%
	200	48.61 ± 3.53[a2b1]	33.10%
	50	51.91 ± 6.02	28.56%
CSB	100	48.59 ± 5.59	33.13%
	200	45.65 ± 8.90[a1]	37.17%

Values are presented as mean ± SEM and analyzed using a One-way ANOVA followed by Tukey's Post hoc test; n = 5; Control received 2%Tween 80; IP, imipramine; CSE, *Carissa spinarum* Linn. ethylacetate fraction; CSB, *Carissa spinarum* Linn. n-butanol fraction; [1:] $p < 0.05$; [2:] $p < 0.01$; [3:] $p < 0.001$; a, compared to control; b, compared to imipramine; c, compared to 50mg/kg; d, compared to 100mg/kg; e, compared to 200mg/kg.

4.6. Evaluation of Possible Mechanisms of Action

According to the results of the antidepressant-like activity in the current study, CSE was found to be the most active fraction. Hence, this fraction was used to investigate the possible mechanism (s) of action involved using different pharmacological agents.

The effect of various drugs on the antidepressant-like activity of CSE using the FST model is presented in Fig. 2. Pretreatment with yohimbine, sulpiride, atropine, and L-Arginine significantly ($p < 0.05$) reversed the antidepressant-like effect of CSE50. By contrast, pretreatment with prazosin, cyproheptadine, and methylene blue did not have effect on the antidepressant-like activity of CSE50. Among the agents that reversed the effect, only sulpiride and L-arginine were capable of returning duration of immobility to the controls level.

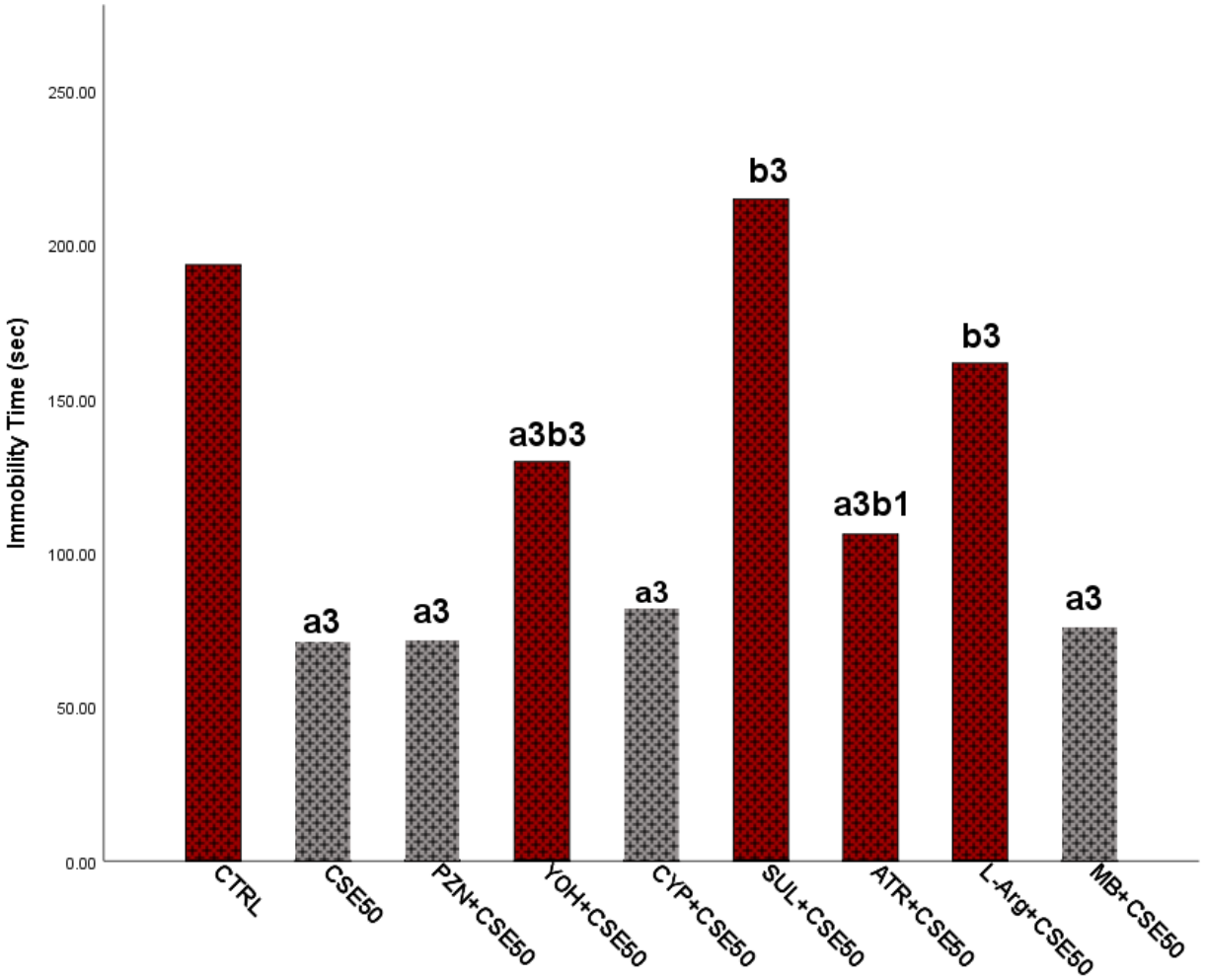

Fig 2: The effects of various pharmacological drugs on the antidepressant-like effect of ethyl acetate fraction of the root bark of *C. spinarum* using forced swim test in rats; Data are presented as mean ± SEM and analyzed using a One-way ANOVA followed by Tukey's post hoc test; n=6; Control received normal saline (10 ml/kg); CSE50, *Carissa spinarum* Linn. ethyl acetate fraction at 50 mg/kg; PZN, Prazosin; YOH, Yohimbine; CYP, Cyproheptadine; SUL, Sulpiride; ATR, Atropine; L-Arg, L-Arginine; MB, Methylene blue; 1: $p < 0.05$; 2: $p < 0.01$; 3: $p < 0.001$; a, compared to control; b, compared to CSE50.

4.7. Quantification of phytochemical constituents

4.7.1. Total flavonoids content

TFC content was estimated by Aluminum chloride colorimetric method using quercetin as a standard using an equation (Y = 0.02413*X - 0.01900) with a correlation coefficient (R^2) of 0.9986 (Fig. 3). Accordingly, TFC was found to be 12.43 mgQE/g and 2.07 mgQE/g CSE and CSB, respectively.

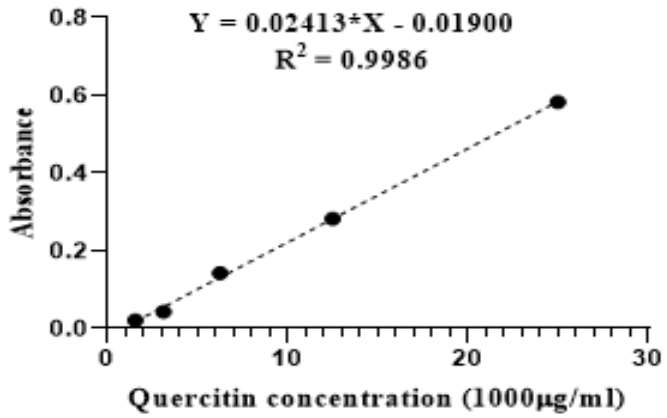

Fig 3: Calibration curve for Quercetin standard solution

4.7.2. Total phenols content

TPC content was estimated by Follin-Ciocalteu method using Gallic acid as a standard. Based on the calibration curve (Fig 4), an equation (Y = 0.009296*X + 0.05072) with a correlation coefficient (R^2) of 0.9997 was used to calculate the concentration of TPC. Accordingly, it was found to be 42.42 mgGAE/g and 29.8 mgGAE/g of CSE and CSB, respectively.

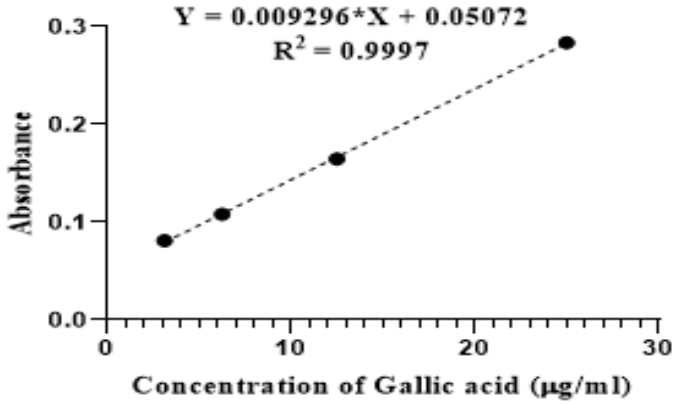

Fig 4: Calibration curve for Gallic acid standard solution

4.7.3. Total alkaloids content

TAC content was estimated using atropine as a standard. A calibration curve (Fig. 5), with an equation of Y = 0.002184*X + 0.03608 and a correlation coefficient (R^2) of 0.9935 was used to determine the concentration of TAC. Accordingly, CSE and CSB were found to be containing 0.17 mgATE/g and 0.07 mgATE/g of TAC, respectively.

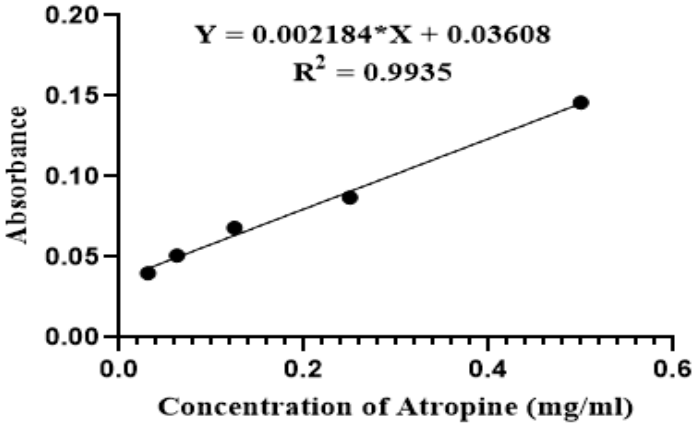

Fig 5: Calibration curve for Atropine standard solution

As described above, TFC, TPC and TAC contents were higher in CSE than CSB. In addition, TAC was lower in both fractions compared to TFC and TPC, the order being TPC > TFC > TAC.

5. DISCUSSION

The present study attempted to evaluate the antidepressant-like activity and explore the possible mechanism (s) of solvent fractions of a root bark of a plant claimed to be used for the management of depression in Ethiopia (Moravec et al., 2014).

Several experimental models are essentially used to assess and evaluate the efficacy and potency of agents with antidepressant-like activity. Among those, FST (Despair behavior test) and TST were used in this study due to their ease of use, high reliability, and specificity, as well as for their sensitivity to all classes of antidepressant treatments (Cryan et al., 2002). Muricidal behavior test was also used to determine the antidepressant-like effect of solvent fractions on the aggressiveness behavior in rats that resembles a state of depression in humans (Brown et al., 1979). Additionally, OFT was used to rule out any unspecific locomotor effect of the solvent fractions of root bark of *C. spinarum* (Gould et al., 2009).

In the present study, oral administration of the solvent fractions of a root bark of *C. spinarum* at different doses indicated that CSE and CSB are endowed with antidepressant-like activity in the rodent models of depression used in the present study. However, CSA did not show any appreciable antidepressant-like activity as reported elsewhere (Ya'u et al., 2017). Moreover, a non-significant increment in the total number of squares scored in OFT with CSE could be an indicator for some anxiolytic effect. However, there was no apparent effect on the locomotor activity with CSE and CSB, demonstrating that the effect is not due to non-specific psychostimulant activity of the fractions.

Depression as a stress byproduct or a stress-related disorder, initiates a neuronal, endocrine, and behavioral responses. In FST and TST, rodents are exposed to an inescapable situation, which leads to endocrine disturbances and alteration in the corticosterone level, as a result of activating the major neuroendocrine stress system, the HPA axis (Bangasser and Valentino, 2014; Brigitta, 2002). Thus, corticosterone level can be used as a possible marker of depression. CSE, particularly in the middle and higher doses, consistently reduced corticosterone level in both TST and FST, although such consistent reduction was not observed with CEB. This effect provides additional evidence

for the antidepressant-like activity of the fractions and CSE is endowed with better activity than CSB.

The antidepressant-like activity of CSE and CSB observed in the present study could be attributed to the presence of phenols, flavonoids, and alkaloids in the fractions. Many scientific studies have reported that these secondary metabolites are highly available in plants with antidepressant-like activity (Akinpelu et al., 2017; Malik et al., 2020). Quantitative analysis demonstrated that CSE had more secondary metabolites than CSB, at least, in part, explaining the difference in activity between the two fractions. The difference in polarity of the solvents used for fractionation could form the basis for the selective distribution of the secondary metabolites into the CSE than CSB. These secondary metabolites are thought to exert their antidepressant-like effect individually or in synergy via interacting with different neurotransmitters involved in the neurobiology of depression. For instance, flavonoids such as quercetin and kaempferol have been detected as an active constituents of *C. spinarum* (Al-Youssef and Hassan, 2014) and these flavonoids are reported to possess antidepressant-like activity (Park et al., 2010). Alkaloids have been found to decrease the immobility behavior in FST and several preclinical studies have demonstrated their antidepressant-like activity (Al-Youssef and Hassan, 2014), although their concentration was found to be very low in *C.spinarum*. Phenols, flavonoids (polyphenol), and alkaloids are among the most widely studied secondary metabolites whose mechanisms could involve stimulation of 5-HT, NE, DA, and GABAergic neurotransmission systems (García-Ríos et al., 2020; Kennedy and Wightman, 2011; Malik et al., 2020).

As CSE was found to be the most active fraction in the host of depression paradigms used in the present study, further investigation of the possible mechanism (s) was conducted using this fraction based on several hypotheses proposed to explain the pathophysiology of depression.

α2-adrenoceptors are inhibitory auto-receptors that autoregulates the NE system. The affinity and density of these receptors are highly increased in depressed patients (Maletic et al., 2017), thus activation of the adrenoceptors has a critical effect on the behavioral

activation and function of the target neuron (Stone et al., 2003). Thus, dysregulation of α2-adrenoceptors is associated with depression (Cottingham and Wang, 2012). The fact that yohimbine but not prazosin that was able to reverse the antidepressant-like effect of CSE suggests that the fraction mediated its effect via α2-but not α1-adrenoceptors. In line with our findings, previous studies also reported that prazosin was unable (Bettio et al., 2011; Mensah, 2016) but yohimbine was able (Abiola et al., 2019; Ishola et al., 2013) to reverse the antidepressant-like effect of plant extracts. These studies collectively indicate that plant extract may preferentially interact with α2-adrenoceptors to produce antidepressant-like effect. Indeed, studies indicate that some flavonoids interact with α2-adrenoceptors, which produce depression-like symptoms in animals (Kaur et al., 2007), while some flavonoids show antidepressant-like activity (Butterweck et al., 2000).

Pretreatment with cyproheptadine did not reverse the antidepressant-like effect of CSE, indicating that the effect was not mediated via the 5-HT_2 receptor neurotransmissions. Similar findings were reported elsewhere (Ishola et al., 2012). The dopaminergic system is a highly important target implicated in regulating depression through its important roles in processing the reward stimuli, cognition, mood, attention and learning (Dunlop and Nemeroff, 2007; Ploski and Vaidya, 2021). Pretreatment with sulpiride significantly reversed the antidepressant-like effect of CSE, suggesting the effect could involve the dopaminergic system. Indeed, several studies have suggested the role of sulpiride in altering the antidepressant-like activity of different plant extracts in rodents (Bettio et al., 2011; Ishola et al., 2015) as well the influence of some flavonoids on the dopaminergic system (Pannu et al., 2021; Wang et al., 2010).

The cholinergic system plays a major role in regulating CNS functions such as arousal, attention, cognition and memory. The cholinergic neurons are connected with the hippocampus and the VTA and participate in the reward system functionality and mood regulation. Impairment of this system accounts for the development of cognitive symptoms observed in patients with depression. Pretreatment with atropine significantly reversed the antidepressant-like effect of CSE, indicating that the muscarinic cholinergic system is involved in the antidepressant-like effect of the fraction. Similar observations were made in other studies (Ishola et al., 2015; Owope et al., 2016) and some studies

indicated that the antidepressant-like effect of some isolated flavonoids are reversed by atropine and suggesting the involvement of the cholinergic system in their effect (Onasanwo et al., 2015).

The involvement of nitric oxide in the physiological neural function such as synaptic plasticity including depression and neurological disorders is well recognized (Dhir and Kulkarni, 2011; Zhou et al., 2018). NO is formed by the enzyme NO synthase from L-arginine and inhibited by L-arginine analogs. Pretreatment with L-arginine reversed the antidepressant-like activity of CSE, indicating the involvement of NO in the activity of the fraction. NO is thought to have a dual role in modulation of depression in TST and FST, as its synthesis or inhibition of its synthesis is shown to produce antidepressant-like effect (da Silva et al., 2000; Inan et al., 2004). Although L-arginine is generally believed to enhance antidepressant-like activity of various agents, there are instances where other effects have been reported. For example, moderate doses of L-arginine produce antidepressant-like effect but higher doses are devoid of any activity (da Silva et al., 2000). Moreover, co-administration of L-arginine with effective dose of modafinil has been shown to attenuate the antidepressant-like effect of modafinil (Omidi-Ardali et al., 2021). Thus, the attenuation of CSE effect with L-arginine could either be related to high dose (750 mg/kg) or co-administration with effective dose of CSE. In a parallel experiment, pretreatment with methylene blue (an inhibitor of nitric oxide synthase and an inhibitor of soluble guanylate cyclase (sGC)) did not reverse the effect of the antidepressant-like effect of CSE. Contrary to our findings, reports in the literature show the antidepressant-like effect of methylene blue in FST (Delport et al., 2017; Heiberg et al., 2002), which could not be replicated in the present study. Such discrepancies could be related to the multiple role of NO in depression and achieving as optimum concentration of nitrites in the brain during treatment with agents acting via this pathway to produce antidepressant-like activity (Omidi-Ardali et al., 2021).

Although this study revealed that the adrenergic, dopaminergic, cholinergic and NO might be involved in the mechanism of action of the antidepressant-effect of CSE, only L-arginine and sulpiride were capable of returning duration of immobility to the control

level, suggesting dopaminergic and NO might be the most likely mechanisms by which CSE mediated its action.

6. LIMITATION OF THE STUDY

Individual variation and sample size could be factors that contribute to variations observed in the measurement of corticosterone level. Measuring brain corticosterone than serum level could have given a better picture. Nevertheless, the use of several models for the study could offset these limitations.

7. CONCLUSIONS

The results from this study demonstrated that CSE and CSB of the root bark of *C. spinarum* are endowed with antidepressant-like effects in TST and FST, as well as the muricidal test, with the former seemingly more active than the latter. This was further augmented by a reduction of serum corticosterone levels. The study indicated that the antidepressant effect of CSE might be mediated through α2-adrenergic, D2-dopaminergic receptors, muscarinic cholinergic system, as well the L-arginine-NO pathway. The quantification study suggests that flavonoids, phenols, and alkaloids present in the solvent fractions could be responsible for the observed activity.

8. SUGGESTION FOR FUTURE WORK

Based on the findings of the present study the following recommendations are proposed:

- Isolation of the pure compounds (secondary metabolites) should be carried out to establish the active compound responsible for the activity of the experimental plant.
- Using other models of depression should be done to confirm the antidepressant properties of the solvent fractions and the isolated active compounds.
- Other assays should be undertaken to explore how the active compounds interact with specified neurotransmitter and hormones contribute in the neurobiology of depression.

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